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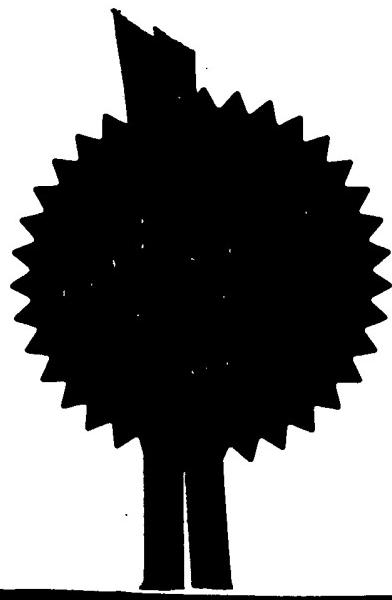
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2. Patent application number

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3. Full name, address and postcode of the or of
each applicant (underline all surnames)

University of Leicester

University Road
Leicester LE1 7RH

Patents ADP number (if you know it)

Great Britain

00798348001

4. Title of the invention

Novel Molecule

5. Name of your agent (if you have one)

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"Address for service" in the United Kingdom
to which all correspondence should be sent
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Regent House
Heaton Lane
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Country

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Number of earlier application

Date of filing
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8. Is a statement of inventorship and of right
to grant of a patent required in support of
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Description	12
Claim(s)	3
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Request for preliminary examination and search (*Patents Form 9/77*)

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Date

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Novel Molecule

The present invention concerns bacterial autoinducers of growth, methods for their purification, autoinducers purified by such methods, and their use to induce the growth of bacteria, both the source organism and other species.

Signalling events between bacteria and host cells are an integral component of the dynamic and complex process of infection and disease. It has recently become clear that signalling between bacteria is also of importance to this process.

Low molecular weight, diffusible signal molecules produced by bacteria, termed autoinducers (AI), play a crucial role in the development of bacterial infections, of both plants and animals. These autoinducers may determine whether or not an initial infection, often involving only a very few bacteria, will succumb to the many defence mechanisms of a host or whether these host defences are overcome, and bacterial growth and disease occur.

One class of autoinducers has already been well-characterised, the N-acyl homoserine lactones, which are composed of derivatives of amino acid and fatty acid molecules. This family of molecules play a key role in the mechanisms by which Gram negative bacteria monitor population densities, factors which are important in virulence of a number species. However, despite the fact that N-acyl homoserine lactone-type sensing systems have been shown to exist in *E. coli*, there is so far no evidence that N-acyl homoserine lactones themselves are made by, or play a role in the pathogenesis of this organism. In addition, no evidence has been so far been presented to suggest a role for these autoinducers in the pathogenesis of *Salmonella*.

The existence of an additional class of autoinducer molecule has been shown, the AI being different from the homoserine lactones. These also appear to play an important role in pathogenesis.

A purported bacterial AI was isolated by Lyte, M. *et al.* (1996, FEMS Microbiology Letters, 139: 155-159) having a molecular weight of approximately 10,000 Da (see also, Lyte, M., 1993, Journal of Endocrinology, 137: 343-345; US 5,629,349).

The present inventors have succeeded in isolating, purifying and characterising a novel autoinducer from *E. coli*.

According to the present invention there is provided a bacterial autoinducer, characterised in that it has substantially the following properties:

- i) it has an apparent molecular weight of 300-400 daltons when eluted in 0.15M NaCl;
- ii) it has an apparent molecular weight of 400-500 daltons when eluted in 0.50M NaCl;
- iii) it has a substantial negative charge;
- iv) it is polar;
- v) it is heat-stable;
- vi) it is stable to lyophilisation;
- vii) it induces at least *E. coli*, *Campylobacter jejuni*, *Salmonella enteriditis*, *Yersinia enterocolitica*, *Proteus mirabilis* and *Enterococcus faecalis*; and
- viii) it has no substantial absorbance at 280 nm;

The bacterium may be *E. coli*.

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The bacterium may be *Salmonella*, for example *S. enteriditis* or *S. typhimurium*.

The *E. coli* autoinducer is a low molecular weight diffusible signal molecule, initially found as a bacterial response to physiologically relevant concentrations of noradrenaline, such as those found in the gastrointestinal tract of mammalian hosts. This effect is not nutritionally mediated. The half-life of activity of intestinal nor-adrenaline is quite short lived - the hormone is active for only a few hours, before becoming irreversibly sulphonated. However, this transient exposure to nor-adrenaline is sufficient to induce the bacteria to synthesize their own growth stimulus, the autoinducer, which has much greater stability. The autoinducer acts by effecting both accelerated growth rate, increased bacterial cell numbers and the production of virulence factors, such as toxins and adhesins, the activity being cross-species specific.

The apparent molecular weight of the molecule is dependent upon the elution conditions used (see 'Experimental' below), due to the substantial charge the molecule has. Experiments (below) have shown the charge on the molecule to be greater than that on ATP. The molecule has also been found to be polar. It is heat stable and is capable of being autoclaved at 121 °C. Similarly it is capable of withstanding lyophilisation. The molecule is also capable of inducing cross-species stimulation

Also provided according to the present invention is a method for isolating and purifying a bacterial autoinducer from a sample comprising the steps of:

- i) collecting a sample containing the autoinducer;
- ii) fractionating the sample to give fractions corresponding to molecular weights of approximately 300-600 Daltons; and
- iii) purifying the sample using anion-exchange chromatography.

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It may comprise the additional step of concentrating the sample prior to fractionation.

Concentration may be achieved by means of ultrafiltration. Such ultrafiltration may be performed with a membrane molecular weight cut-off (MWCO) of approximately 100 Daltons. Alternatively, concentration may be by means of lyophilisation or filtration or a combination thereof.

The sample may be collected from a culture containing bacteria and the autoinducer. It may be a supernatant collected from a centrifuged culture containing bacteria and the autoinducer.

Fractionation may be by means of size exclusion gel filtration.

Size exclusion gel filtration may be performed using a buffer of approximately 100mM ammonium bicarbonate, pH 8.0, anion exchange purification being performed on a Mono P anion exchange column with a triethylammonium bicarbonate gradient.

Alternatively, size exclusion gel filtration may be performed using a buffer of approximately 20 mM potassium phosphate containing 150 mM NaCl, pH 7.4, anion exchange purification being performed on a Mono Q anion exchange column with a NaCl gradient.

Size exclusion separation of the autoinducer may also be performed using preparative ultrafiltration with a MWCO greater than that of the autoinducer, for example 3000 Da.

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Other conditions for performing anion-exchange purification and concentration of the sample will be readily apparent to one skilled in the art, particularly with regard to the highly distinctive physical characteristics of the autoinducer.

Also provided according to the present invention is a bacterial autoinducer isolated and purified according to the method of the invention.

Also provided according to the present invention is the use of a bacterial autoinducer according to the present invention in inducing bacterial growth, the production of bacteria toxins or the production of bacterial adhesins. The use may of course be with bacteria of the species from which the autoinducer was derived, or of another species.

The invention will be further apparent from the following examples which show by way of example only, methods of isolating and purifying bacterial autoinducers.

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Experimental

Extraction and Purification

1) Manufacture in *E. Coli*

E.coli 0157:H7 bacteria (approximately 50-500 CFU/ml) are inoculated into SAPI minimal medium (6.25 mM NH₄NO₃, 1.84 mM KH₂PO₄, 3.35 mM KCl, 1.01 mM MgSO₄ and 2.77 mM glucose, pH 7.5) supplemented with 30% (v/v) Adult Bovine Serum (Sigma), and 1% (v/v) previously made *E. coli* 0157:H7 autoinducer. The cultures are grown statically for 24 hours at 37°C, in a 5% CO₂ incubator. The bacteria are pelleted by centrifugation, and the culture supernatants containing the autoinducer are sterilised by filtration through a 0.2μm diameter filter.

The production of autoinducer in SAPI/serum media is also achieved using growth stimuli of nor-adrenaline (50 μM), ammonium sulphate (40 mM), yeast extract (Oxoid) (0.005% w/v), or non-physiologically relevant levels of dopamine or adrenaline. Autoinducer production is also induced by the replacement of Bovine serum by 5% (w/v) casein hydrolysate (Oxoid). Under these culture conditions, no nor-adrenaline, autoinducer or other growth stimulus addition is required to initiate bacterial growth and autoinducer synthesis. Growth conditions for all of these alternative methods of induction are as above.

Other strains of *E. coli*, including enteropathogens and laboratory strains, are able to manufacture autoinducer. Several species of *Salmonella* (*S. enteriditis* and *S. typhimurium*) are also good sources of AI.

2) Purification

Gel filtration Chromatography

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The autoinducer supernatants are lyophilised, and re-dissolved at 1/5 of their original volume in distilled water, and re-filtered. These are then fractionated (2ml of X 5-concentrated material) using gel filtration (size exclusion) chromatography on two Superdex pep HR10 columns (total volume 50 ml) connected in series to a Pharmacia FPLC (Fast Protein Liquid Chromatography system).

The chromatography buffer is either:

- 1) 100 mM ammonium bicarbonate, pH 8.0 for AI samples destined for purification using Mono P (Pharmacia) anion exchange chromatography (below); or
- 2) 20 mM potassium phosphate containing 150 mM NaCl, pH 7.4 for samples of AI destined for purification using Mono Q (Pharmacia) anion exchange chromatography (below).

With either buffer system, the *E. coli* autoinducer is eluted in fractions corresponding to molecular weights of between 600 and approximately 300 Da, average about 400-500 Da (typically elution volumes, V_e of 32-50 ml).

Molecular weight estimations by gel filtration are determined by comparing the volume required to elute a molecule from a column (V_e) with the volume required to elute standard proteins of known molecular weight. The V_e of the standard proteins is plotted against the \log_{10} of their molecular weights, and the resulting graph is used to estimate the molecular masses of unknown molecules based on their V_e values. In gel filtration separation, molecules are eluted in order of decreasing size.

Approximate molecular weights only are given for the autoinducer because the extreme electronegativity of the autoinducer causes it to interact with the gel filtration column in a manner which is charge-dependent, as well as size dependent. This leads to anomalous

retardation of the molecule, an anomalously increased V_e and a corresponding underestimation of molecular weight. This has been demonstrated by comparing the average molecular weight of the AI fractionated in buffer containing 150 and 500 mM NaCl. With the higher salt concentration, the charge effect is nullified, separation parameters are now more truly of size only, and the autoreducer elutes earlier from the column, with an increased apparent molecular weight of around 400-500 Da, compared to 300-400 at the lower NaCl concentration. However, concentrations of salts higher than those already specified for the gel filtration fractionation are not used, since they would severely interfere with the subsequent anion exchange step of the purification. More precise estimations of the molecular weight of the autoreducer can be obtained using Mass Spectrometry.

Size separation is also achieved using a preparative ultrafiltration cell, using a membrane with a MWCO of for example 3000 Da. Similarly, the initial pre-gel filtration concentration, currently lyophilisation, can also be performed using ultrafiltration, using a membrane with a MWCO of 100 (to retain the AI). However, the high initial concentrations of serum proteins in the crude autoinducer preparation does result in the production of highly viscous, difficult to handle solutions, which can potentially result in overall loss of AI activity, if this method is used as a means of pre-fractionation concentration.

Anion Exchange

The next step of the purification, anion exchange is achieved in one of two ways:

- 1) By using a weak anion exchanger, Mono P and a volatile salt (triethyl ammonium bicarbonate - TEAB) to elute the autoinducer.
 - 2) By using a strong anion exchange column, Mono Q 5/5, and a non-volatile salt (sodium chloride) to elute the autoinducer.

Mono P Anion Exchange Chromatography

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The pooled Superdex fractions are loaded onto a Pharmacia Mono P weak anion exchange column (equilibrated in 20 mM triethylammonium bicarbonate (TEAB) buffer, pH 7.5). The autoinducer is purified using a 30 ml gradient of (20 to 1000) mM TEAB, pH 7.5. The autoinducer elutes between 500 and 700 mM TEAB. The TEAB buffer is removed from the AI fractions by lyophilisation (around 2 freeze-drying treatments are sufficient).

Mono Q Anion Exchange Chromatography

When using this procedure, the Mono Q column is equilibrated in 20 mM potassium phosphate, pH 7.2, and residual serum proteins removed by a 10 ml linear gradient of 0-700 mM NaCl, followed by 5 ml of 700 mM NaCl. The autoinducer is removed from the column by a step-wise salt increase to 2 M NaCl. The autoinducer typically elutes 2 ml after the step up to 2 M NaCl, in a volume of around 6-8 ml.

The sodium chloride can be removed from Mono Q fractionated autoinducer by re-gel filtration in 100 mM ammonium bicarbonate as described above (0.5 - 0.7 ml of NaCl-containing sample, per 50 ml column set-up), although the presence of the salt in such preparations of autoinducer does not limit its suitability for the general purpose of stimulating growth of bacteria. The small size of the autoinducer and, once purified, its reduced stability to prolonged exposure to extremes of acidity (1-2 M HCl), means that alternative methods of de-salting, such as Dowex 50 (Sigma) cation exchange, or dialysis are unsuitable. Ultrafiltration as a means of simultaneous de-salting and concentration is also possible, although solvent passage and therefore rates of concentration, are extremely slow indeed (around 0.001 ml per hour per cm² of membrane).

Alternative methods of purifying the autoinducer have been examined, such as organic solvent extraction (this has been tried with preparations of autoinducer from all stages of its purification, both before and after reduction in electronegativity by acidification), and HPLC (High Pressure Liquid Chromatography) Reverse Phase chromatography (C8

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and C18 columns; Vydac) using various solvent systems and ion-pairing reagents. Because of the very electronegative, polar nature of the autoinducer, none of these procedures are successful. It is for these reasons that attempts made to isolate the autoinducer using published methods of N-acyl homoserine lactone extraction and purification were also unsuccessful.

The *E. coli* autoinducer of growth has also been shown to bind to a protein in the SAPI/serum medium in which it is manufactured. It is possible to extract the autoinducer from this protein by appropriate chemical treatment. The SAPI/serum autoinducer preparation undergoes gel filtration fractionation in 20 mM potassium phosphate containing 150 mM NaCl, pH 7.4 (as described above) and serum proteins, which elute as a broad peak, are collected in fractions corresponding to elution volumes (V_e) of 14-22 ml. These fractions are then diluted 1:5 in 20 mM potassium phosphate containing 300 mM NaCl, pH 7.4 and heated at 95-100 °C for 30 minutes. This results in the release of the autoinducer into the supernatant. Precipitated proteins are removed by centrifugation, and the supernatant containing the autoinducer further purified using anion exchange chromatography (as described above).

Properties of the *E. coli* Autoinducer of Growth

Growth Stimulation and Virulence Factor Production

The *E. coli* autoinducer has been found to be effective at very low levels in stimulating growth in serum/SAPI medium by a factor of over 100,000 relative to control cultures. This is comparable to levels below detectability by UV-spectroscopy, indicating a substance with hormone-like properties. The AI can stimulate growth of an extremely low initial inoculum (80 CFU/ml) to a final level of over 10^8 CFU/ml. It can also stimulate the increased production of toxins and adhesins, important in the pathogenesis of bacterial infection, to readily detectable levels in as little as 6 hours.

Stability

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The *E. coli* autoinducer is a very stable molecule. It is especially resistant to heat inactivation, at all stages of its purification. It can even be autoclaved at 121°C without losing activity. It is also stable to very prolonged storage in serum. SAPI solution, without any loss of growth stimulation capability, e.g. at least 24 weeks, 4°C; at least 10 months, -20°C. It is also completely stable to lyophilisation, and to storage in a dried, powder state (at least 12 weeks at 4°C).

The purified autoinducer also shows some stability to acid conditions (pH 2 or less), although less than AI in serum/SAPI medium. Anion exchange-purified autoinducer retains around 50% of its activity after 4 hours of 1% TFA (Trifluoro acetic acid) treatment, or around 10% after 1-2 hours of 1-2 M HCl treatment.

Size

Dialysis and gel filtration chromatography suggest a molecular weight of around 400-500 Da.

Charge

The *E. coli* autoinducer appears to a very negatively charged, very polar molecule.

Spectroscopy

Preliminary wavelength scans have shown that the autoinducer has no significant absorbance at 280 nm, a wavelength typically associated with proteins, but does seem to have absorption maxima at around 206 - 212 nm, and possibly 250 - 260 nm.

Cross-species Stimulation

The autoinducer produced by *E. coli* has also been found in serum/SAPI medium to stimulate growth to an enhanced level of a range of other bacteria. To date, the following major human pathogens have been found to be induced by the *E. coli* autoinducer:

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Campylobacter jejuni, *Salmonella enteriditis*, *Yersinia enterocolitica*, *Proteus mirabilis* and *Enteroccus faecalis*.

In addition, *Salmonella*, the gastroenteritis pathogen, *Proteus*, a major urinary tract (UTI) pathogen, and *Enterococcus faecalis*, a UTI pathogen and a causative agent of endocarditis, are also able to synthesize autoinducers of their own, which are also capable of stimulating the growth of *E. coli* and of each other.

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CLAIMS

1. A bacterial autoinducer having substantially the following properties:
 - i) it has an apparent molecular weight of 300-400 daltons when eluted in 0.15M NaCl;
 - ii) it has an apparent molecular weight of 400-500 daltons when eluted in 0.50M NaCl;
 - iii) it has a substantial negative charge;
 - iv) it is polar;
 - v) it is heat-stable;
 - vi) it is stable to lyophilisation;
 - vii) it induces at least *E. coli*, *Campylobacter jejuni*, *Salmonella enteriditis*, *Yersinia enterocolitica*, *Proteus mirabilis* and *Enterococcus faecalis*;
 - viii) it has no substantial absorbance at 280 nm; and
2. A bacterial autoinducer according to claim 1, being an *E. coli* autoinducer.
3. A method for isolating and purifying a bacterial autoinducer, comprising the steps of:
 - i) collecting a sample containing the autoinducer;
 - ii) fractionating the sample to give fractions corresponding to molecular weights of approximately 300-600 Daltons; and
 - iii) purifying the sample using anion-exchange purification.
4. A method according to claim 3, comprising the additional step of concentrating the sample prior to fractionating.

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5. A method according to claim 4, concentration being achieved by passing the sample through an approximately 0.2 µm diameter filter, lyophilising the sample and passing it through an approximately 0.2 µm diameter filter.

6. A method according to claim 4, concentration being achieved by means of ultrafiltration.

7. A method according to claim 6, ultrafiltration being performed with a molecular weight cut-off of approximately 100 Daltons.

8. A method according to any one of claims 3-7, the sample being collected from a culture containing bacteria and the autoinducer.

9. A method according to claim 8, the sample being a supernatant collected from a centrifuged culture containing bacteria and the autoinducer.

10. A method according to any one of claims 3-9, size exclusion gel filtration being performed using a buffer of approximately 100 mM ammonium bicarbonate, pH 8.0, anion exchange purification being performed on a Mono P anion exchange column and triethylammonium bicarbonate.

11. A method according to any one of claims 3-9, size exclusion gel filtration being performed using a buffer of approximately 20 mM potassium phosphate containing 150 mM NaCl, pH 7.4, anion exchange purification being performed on a Mono Q anion exchange column and NaCl gradient.

12. A method according to any one of claims 3-11, the bacterium from which the autoinducer is derived being *E. coli*, *S. enteriditis* or *S. typhimurium*.

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13. A bacterial autoinducer isolated and purified according to the method of any one of claims 3-12.

14. A bacterial autoinducer according to any one of the preceding claims used to induce bacterial growth, the production of bacterial toxins or the production of bacterial adhesins.